Structure and Role of the Terminal Repeats of Epstein-Barr Virus in Processing and Packaging of Virion DNA

JULIE ZIMMERMANN AND WOLFGANG HAMMERSCHMIDT*

*Institut für Klinische Molekularbiologie und Tumorgenetik, GSF-Forschungszentrum
für Umwelt und Gesundheit GmbH, D-81377 Munich, Germany*

The linear virion Epstein-Barr virus (EBV) DNA is terminated at both ends by a variable number of direct, tandemly arranged terminal repeats (TRs) which are approximately 500 bp in size. The number of TRs at each terminus can vary. After infection of host cells, the EBV DNA circularizes via the TRs by an unknown mechanism, and replication of the viral DNA during the lytic phase of the EBV life cycle leads to large DNA concatemers which need to be cleaved into virion DNA units, eventually. This cleavage event occurs at an unknown locus within the TRs of EBV, which are the *cis*-acting elements essential for cleavage of the concatemers and encapsidation of the virion DNA. To investigate the mechanism of DNA processing during genome circularization and cleavage of concatemeric DNA, the genomic termini of EBV were cloned, sequenced, and analyzed by direct labeling of the virion DNA. Both termini ended with identical 11-bp elements; the right end has acquired an additional 9-bp stretch that seemed to originate from the leftmost unique sequences. The left terminus is blunt, whereas the right terminus appears to have a 3′ single-base extension. In a transient packaging assay, a single terminal repeat was found to be sufficient for encapsidation of plasmid DNA, and mutagenesis of the TR element defined a region of 159 bp, including the 11-bp element, which is essential for packaging. These results indicate that the genomic termini of EBV are not generated by a simple cut of a hypothetical terminase. The mechanism for cleavage of concatemers seems to involve recombination events.

The termini of Epstein-Barr virus (EBV) virion DNA are involved in various processes of the viral life cycle, which consists of a latent and a lytic phase. Shortly after infection of B lymphocytes, the linear virion molecules are circularized. During the latent phase, the EBV genome is maintained as a circular molecule and its DNA is replicated via *oriP*, the plasmid origin of DNA replication (26). During the lytic phase, which is induced spontaneously or by expression of the BZLF-1 gene (3, 22), DNA replication is mediated by a different origin of DNA replication, termed *oriLyt* (9). Lytic replication leads to long head-to-tail concatemers which are cleaved to generate virion DNA units which are packaged into viral capsids. The protein or complex of proteins responsible for processing of the EBV virion DNA is called the terminase and has not been identified so far.

The termini of EBV DNA consist of a variable number of directly oriented 538-bp terminal repeats (TRs). The TRs of EBV are involved in the circularization event of the genome, and they are the essential elements for cleavage and packaging of the EBV virion DNA (10). The mechanisms by which these processes are accomplished are not clear. The hypothetical terminase cleaves EBV concatemers into unit-length monomers somewhere within the TRs, but the cleavage and recognition sites of the terminase have not been identified yet. The sequence of the circular EBV genome was determined, and the sequence of the fused termini is known (1). The sequences at the termini of the linear virion DNA of EBV, however, have not been studied in detail so far.

We determined the terminal sequences of EBV by cloning and sequencing the terminal fragments of linear EBV DNA. Surprisingly, a sequence of 11 bp is repeated at both ends in direct orientation. An element of 9 bp derived from the most distal unique region near the left terminus appears at the right terminus. These findings could be confirmed by measuring the lengths of small single-stranded end fragments of linear EBV virion DNA. In addition, the fine structure of the EBV genomic termini was analyzed. The linear form of the EBV genome has two different end structures: the left terminus is blunt, and the right terminus has a 3′ single-base extension. We developed a functional assay that allowed us to test recombinant plasmid DNA for packaging by EBV. Recombinant plasmid DNA containing the *cis* elements for replication was transfected into P3HR1 helper cells and tested for packaging by the products of the endogenous EBV. Functional analysis of the TR sequence led to the observation that a single TR element is sufficient for cleavage and packaging of the plasmid DNA by EBV, and deletion analysis of a single TR element showed that a sequence of 159 bp contains the signals essential for packaging. This finding indicates that the sequence requirement for cleavage and packaging extends beyond the vicinity of the site of cleavage itself.

MATERIALS AND METHODS

Cells and virus. HH514 is a cell line derived from a single cell clone of the Burkitt's lymphoma cell line P3HR1 (16). Cells were grown in RPMI 1640 cell culture medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. The virion DNA of the EBV strain, B95-8, that was used for cloning the terminal fragments of EBV and labeling experiments was a gift from Bill Sugden (Madison, Wisconsin).

* Corresponding author. Mailing address: Institut für Klinische Molekularbiologie und Tumorgenetik, GSF Forschungszentrum für Umwelt und Gesundheit GmbH, Marchioninistr. 25, D-81377 Munich, Germany. Phone: 49/89/7099-506. Fax: 49/89/7099-500. Electronic mail address: hammers@gsf.de.

Cloning of the genomic termini. The strategy of cloning the terminal fragments of EBV is as follows (see Fig. 2). EBV virion DNA was made blunt by treatment with T4 DNA polymerase in the presence of the four deoxynucleotide triphosphates. The AMP adapter with the sequence

    5′-TCGTCCCATGGGAATTCTGGACTTTCTACGCATGATGATGATG-3′
    3′-AGCAGGGTACCCTTAAGACCTGAAAGATGCG-5′
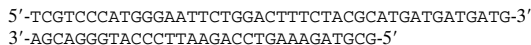
was ligated to the blunt ends. After cleavage of the modified virion DNA with *Bam*HI, the fragments with sizes of 4 to 6 kbp were isolated on preparative agarose gels. Fragments of sizes appropriate for cloning were generated by cleavage of the *Bam*HI fragments with *Nhe*I (left end) or with *Mlu*I (right end). Only the terminal fragments contained the AMP adapter on one side, and they were cloned in defined orientation into the modified pAMP1 vector (Gibco BRL). Clones carrying the left or right terminal fragments were identified by colony hybridization with radioactively labeled probes specific either for the left or for the right end of EBV DNA. An *Eco*RI-*Sal*I fragment with the coordinates 1 to 644 of the EBV(B95-8) DNA cloned into pUC18 (p950) was used for detection of the left terminus; an *Mlu*I-*Bst*NI fragment with the coordinates 169566 to 170117 was cloned into pUC18 (p951) for detection of the right terminus.

5′ and 3′ labeling of the EBV genomic termini. For 5′ labeling, 20 μg of linear native untreated EBV DNA of the B95-8 strain was first dephosphorylated with calf intestine phosphatase and subsequently cut with *Bam*HI after inactivation of the phosphatase. Fragments in the range of 4 to 6.5 kbp, including the end fragments, were recovered from a preparative agarose gel and labeled at the 5′ ends with T4 polynucleotide kinase and 50 μCi of [γ-^{32}P]ATP. These labeled *Bam*HI fragments were cut with enzymes that have unique cleavage sites within the terminal repeat. One half of the prepared fragments was cleaved with *Sau*3AI to generate a left-end fragment of appropriate size, and the other half was cleaved with *Bst*EII to produce the right-end fragment. After cleavage, the DNA was extracted with phenol and butanol and the probes were loaded on an 8% denaturing sequencing gel.

EBV DNA was labeled at the 3′ ends with terminal deoxynucleotidyltransferase (TdT) (Promega), which adds nucleotides to the free 3′ hydroxyl group of the DNA strands. Twenty micrograms of linear, native EBV DNA was melted at 70°C in the presence of spermidin (1 mM) to make the 3′ strands more accessible for the enzyme. After being cooled to room temperature, the DNAs were labeled at the 3′ ends in a volume of 100 μl containing 250 μCi of [α-^{32}P]ddATP, 20 μl of TdT buffer (500 mM cacodylate buffer [pH 6.8]–1 mM CoCl$_2$-0.5 mM dithiothreitol–500 μg of bovine serum albumin per ml), and 2 μl (20 to 40 U) of TdT for 30 min at 37°C. The reaction was stopped by heating of the mixture for 10 min at 70°C. The 3′-labeled DNA was cleaved with *Bam*HI, and the *Bam*HI fragments of between 4 and 6.5 kbp were prepared and cut with *Sau*3AI or *Bst*EII. After extraction with phenol and butanol, the fragments were loaded on an 8% denaturing sequencing gel.

Plasmids. Plasmids p554 and p588 contain the origins of DNA replication, *oriP* and *oriLyt*, and the TRs of the B95-8 strain. The two plasmids were used as positive controls for packaging (10). The negative control p562.3 contains *oriP* and *oriLyt* but lacks the TRs (9). A single copy of the terminal repeats of EBV was isolated with *Sau*3AI or *Eco*NI and cloned into the *Bam*HI site of plasmid p562.3 (pHEBo [26]), resulting in the plasmids p1187 and p1188 (see Fig. 7A). Plasmid p1187 contains B95-8 sequences from nucleotide position 170263 to position 170800; p1188 contains B95-8 sequences from position 170591 to position 171129. Oligonucleotide-directed mutations were introduced into plasmid p1187 as described elsewhere (17) and were confirmed by DNA sequencing. The different mutants which are based on the p1187 plasmid (see Fig. 8) encompass the following deletions: p1438.1a, from 170263 to 170347; p1438.2, from 170348 to 170429; p1438.3, from 170430 to 170507; p1438.4, from 170508 to 170592; p1438.5, from 170593 to 170666; p1438.6, from 170667 to 170737; p1438.7, from 170738 to 170800.

Transient packaging assay. (i) DNA transfections. The plasmids were electroporated into HH514 cells by using the Bio-Rad gene pulser at 960 μF and 220 V. HH514 cells (10^7 cells in a volume of 250 μl of RPMI 1640) were transfected with 10 μg of pCMV BZLF-1 and 10 μg of test plasmid. Four transfections were combined and resuspended in 15 ml of RPMI 1640 with 10% fetal calf serum.

(ii) Preparation of virion DNA from the viral capsids. The supernatant of the transfected cells was harvested after 4 days, and cell debris was removed by centrifugation at 300 × g for 10 min and at 4,000 × g for 20 min. The viral particles were pelleted through a 20% sucrose (in Tris-EDTA-NaCl [TEN]) cushion by centrifugation at 25,000 rpm for 1.5 h at 15°C in an SW28 rotor and resuspended in 900 μl of 0.05% Tween for 1 h at 37°C. To remove cellular DNA contamination, 100 μl of DNase buffer (0.5 M Tris-HCl [pH 7.5]–0.1 M MgSO$_4$–1 mM dithiothreitol) and 30 U of DNase were added and the mixture was incubated for 1 h at 37°C. The viral particles were lysed with 100 μl of proteinase K (10 mg/ml) and 50 μl of 10% sodium dodecyl sulfate overnight at 37°C, and the viral DNA was isolated and purified.

(iii) Analysis of the packaged plasmid DNA. The virion DNA was cleaved either with *Bam*HI or *Hin*dIII in the presence of *Xba*I, which was added to remove contaminating cellular DNA. *Xba*I does not cleave the plasmids but has many restriction sites in cellular DNA. Treatment with *Dpn*I led to the removal of the nonreplicated bacterial input DNA. The restriction fragments were sep-
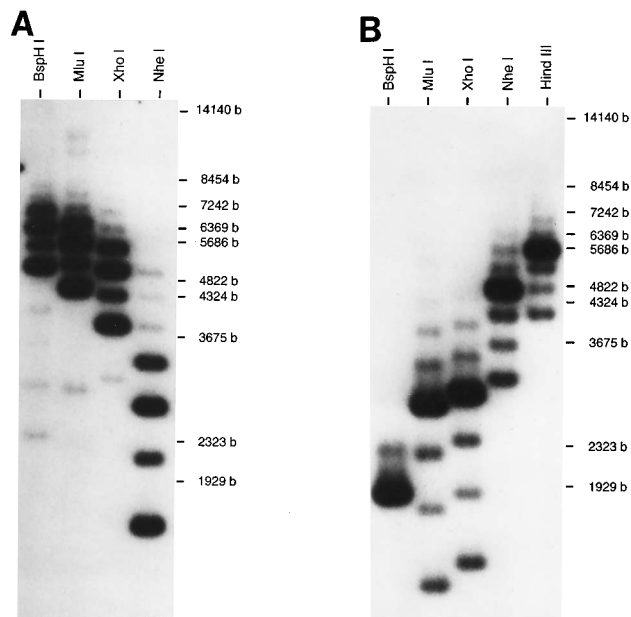

FIG. 1. Restriction analysis of the EBV termini. Linear EBV DNA was cleaved with *Bsp*HI, *Mlu*I, *Xho*I, and *Nhe*I and additionally with *Hin*dIII for the right end. The cleaved EBV fragments were separated by electrophoresis, transferred to nylon filters, and hybridized with probes specific for the left and right ends. (A) Autoradiography of a blot hybridized with radioactively labeled probe containing the unique sequence of the left terminus (strain B95-8, nucleotides 1 to 644). (B) Autoradiography of a blot hybridized with a radioactively labeled probe containing the unique sequence of the right terminus (strain B95-8, nucleotides 169566 to 170117). Four major bands with similar intensities and a size difference of ca. 500 bp represent the left-terminus fragments with one to four TRs. The right-terminus fragment with four TRs has the strongest intensity. After longer exposure, fragments with up to 10 TRs can be detected. The restriction enzymes chosen for cloning the EBV ends were *Nhe*I for the left terminus and *Mlu*I for the right terminus, which produce small fragments of between 1.5 and 3.5 kbp.

arated by electrophoresis on agarose gels and transferred to nylon filters. The filters were hybridized with DNA of vector pUC18, which detected the prokaryotic plasmid DNAs only.

RESULTS

The terminal fragments of linear EBV DNA. To select appropriate restriction enzymes to clone the terminal fragments of EBV DNA, linear EBV(B95-8) virion DNA was digested with a set of various enzymes that do not cleave within the TR sequence. The EBV fragments were then separated on an agarose gel, and the terminal fragments were made visible by hybridization of the Southern blot with probes specific for the left and for the right ends (Fig. 1). A ladder of bands represents the terminal fragments, which vary by an increment of ca. 500 bp according to the number of TRs at each end. The distribution of the band intensities is the same as that reported earlier (12). Four equal populations of molecules have one, two, three, or four TRs at the left end. About 70% of the molecules have four TRs at the right end. The restriction enzymes *Nhe*I (for the left end) and *Mlu*I (for the right end) were chosen for cloning because they produce terminal fragments of convenient sizes (1.5 to 3.5 kbp).

Cloning and sequencing of the genomic termini. Several technically different attempts were made to clone the virion DNA termini molecularly. The highest efficiency for cloning was achieved by using the strategy outlined in Fig. 2. The
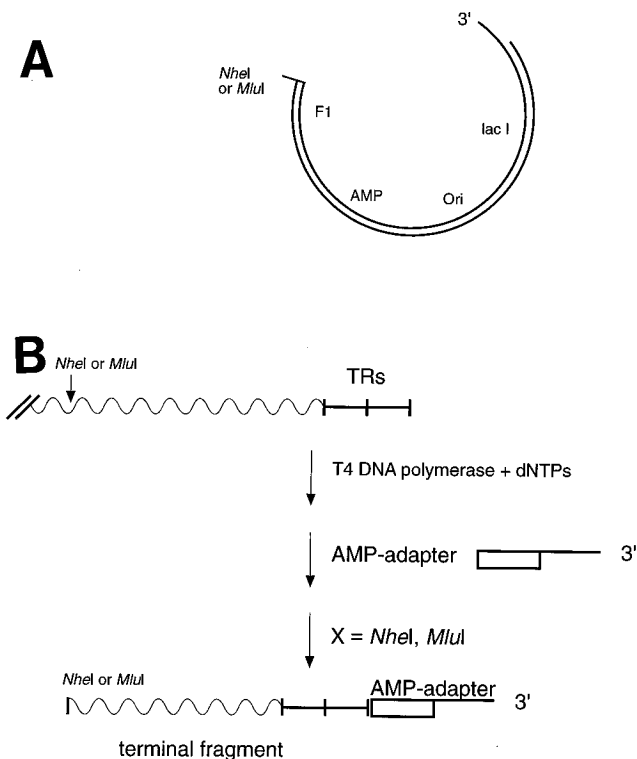
FIG. 2. Cloning strategy for the terminal fragments of EBV DNA. (A) The linearized pAMP 1 vector (Gibco BRL) was chosen for cloning of the terminal DNA fragments. This vector contains 3′ extensions of 12 bases that are not complementary and cannot recircularize unless a fragment with appropriate end structures is inserted. To clone the left and right EBV DNA terminal fragments, the pAMP 1 vector was modified by restriction with *Nhe*I and *Mlu*I, respectively. (B) EBV(B95-8) linear virion DNA was treated with T4 DNA polymerase in the presence of nucleoside triphosphates to provide blunt ends. An AMP adapter complementary to the 3′ extension of the pAMP 1 vector was attached to the termini. Isolated *Bam*HI fragments in a range between 4 and 6.5 kbp were cleaved with either *Nhe*I (left end) or *Mlu*I (right end). The resulting EBV DNA fragments were then cloned into the appropriately modified pAMP 1 vector. Clones containing the terminal fragments were identified by colony hybridization with probes specific for the left and right ends (for details, see Materials and Methods).

pAMP1 vector is a linearized DNA molecule with two 3′ overhangs of 12 bases that are not complementary to each other. The vector recircularizes and becomes transformable into *Escherichia coli* only after a fragment with compatible ends is inserted into it.

To prepare the appropriate inserts, the genomic termini of linear EBV DNA were made blunt and an oligonucleotide adapter for one of the 3′ 12-base overhangs of the pAMP 1 vector was attached. The *Bam*HI EBV fragments of between 4 and 6.5 kbp were cleaved with *Nhe*I (left terminus) or *Mlu*I (right terminus). The AMP adapter was attached to the terminal fragments of EBV only, which could be cloned in defined orientations into the pAMP 1 vector (Fig. 2B).

Clones containing the terminal fragments were identified by colony hybridization with a radioactive probe specific for either the left end or the right end. The junction between vector and insert was sequenced, and the boundaries of left and right termini are summarized in Table 1 and Fig. 3.

Fifteen clones containing the left terminus were analyzed. They all showed the same terminal base pair at position 172241 of the EBV prototype strain B95-8 with the exception of a single clone. Clones with the right fragments were found to be

TABLE 1. Determination of the terminal nucleotides of the EBV DNA end fragments[a]

Coordinate	No. of clones sequenced
Left terminus	
172241	14
172243	1
Right terminus	
172251	13
172250	8
172249	1

[a] Fifteen colonies containing the left terminus and 22 clones containing the right terminus of EBV DNA were sequenced at the junction between vector and insert. With the exception of one clone, all left-end inserts ended at position 172241 of the EBV(B95-8) DNA (1). The terminal base in the right-end clones varied between position 172251 and position 172249. The left cloned termini contained one to three TRs, and all contained the 11-bp element. Two clones derived from the right terminus contained two copies of the TR element; the remaining clones contained only one copy.

heterogeneous, since the EBV inserts terminated at nucleotide positions 172251 to 172249.

The terminal sequences of linear EBV DNA. The sequence analysis of the left and right termini revealed that an 11-bp sequence is duplicated at each end (Fig. 3). This finding was surprising, because other herpesviruses do not show such a terminal sequence redundancy (2, 4, 8, 15, 23). Moreover, the right termini of EBV virion DNA seemed to have acquired a 9-bp unique sequence that appears to originate from the ultimate left boundary of the unique EBV sequence. Figure 4 depicts the arrangements of the 9- and 11-bp elements in the circular EBV genome (1) in comparison with the sequences of the cloned linear termini. The 11-bp element is repeated once in every TR and in addition appears next to the last TR in combination with the 9-bp element. The right termini of EBV end with a sequence of 20 bp (the 9-bp element plus the 11-bp element). All of the left termini end with the 11-bp element. Comparison of the cloned terminal sequences with the published sequence of the fused termini revealed that the right termini could have acquired these 20 bp from the ultimate left end.

Determination of the lengths of terminal fragments in EBV virion DNA. This analysis led to the question of whether these sequence structures do exist at the ends of EBV DNA or whether they result from a cloning artifact. Therefore, the precise lengths of the termini of native, unmodified linear EBV DNA were determined.

B95-8 virion DNA was labeled at the 5′ ends with T4 polynucleotide kinase and [γ-^{32}P]ATP and at the 3′ ends with TdT and [α-^{32}P]ddATP. After digestion with *Bam*HI, the labeled
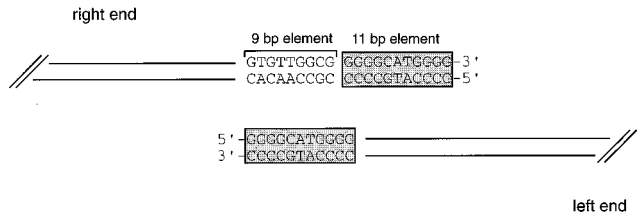

FIG. 3. The terminal sequences at the ends of EBV DNA. Summary of the sequence analyses of the cloned termini revealed that both termini of EBV DNA end with the same 11-bp element. The appearance of the 9-bp element at the right end was unexpected, since this sequence belongs to the unique sequence of the left terminus.
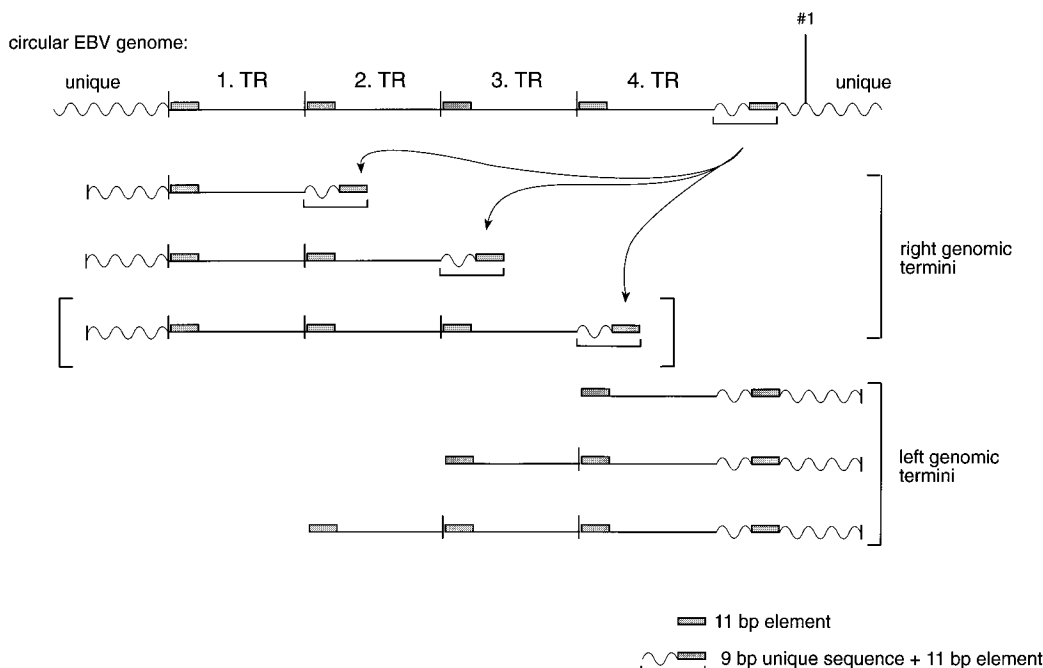
FIG. 4. Comparison of the circular genome and the cloned linear termini of EBV DNA. The upper part of the figure shows the fusion of the EBV DNA termini with four TR elements. Position 1 of the EBV genome is defined by the first *Eco*RI site following the TRs. The 11-bp element is repeated at the beginning of every terminal repeat and once outside the repetitive sequence together with the 9-bp element. In the lower part, the structures of the cloned left and right termini are depicted. The left termini contained one to three TRs and all ended with the 11-bp element. Most right termini had only one TR element, and only 2 of 22 clones contained two TRs. The right-terminus fragments end with a sequence of 20 bp consisting of the 9-bp element and the 11-bp element that seems to be derived from the left end. We assume that the structures of the terminal fragments with three or four TRs are consistent with these findings.

DNA was electrophoresed on an agarose gel and the DNA fragments of 4 to 6.5 kbp, including the terminal fragments, were recovered. The *Bam*HI fragments were then cleaved separately with additional restriction enzymes (the left end with *Sau*3AI and the right end with *Bst*EII) to yield small terminal fragments. These small fragments, end labeled on either the 5′ or the 3′ end, were electrophoresed on denaturing polyacrylamide gels next to sequencing ladders of the corresponding cloned termini as length markers.

As shown in Fig. 5A, the lengths of the expected single-stranded DNA fragments were calculated on the assumption of blunt end structures of the termini. *Sau*3AI digestion of a blunt left terminus would yield two single-stranded fragments labeled at either the 3′ or the 5′ position with a size difference of 5 nucleotides. *Sau*3AI produces a 5′ overhang of 4 nucleotides, and an additional nucleotide is added by labeling of the 3′ end with [α-^{32}P]ddATP. The labeled single-stranded fragments of the right terminus would differ in their lengths by 6 nucleotides because of the additional ddATP nucleotide and the 5′ overhang of five nucleotides derived from the *Bst*EII cleavage site.

The results shown in Fig. 5B showed that the 5′- and 3′-labeled fragments of the left terminus have the predicted lengths (5′ left, 168 bases; 3′ left, 173 bases). The size difference of 5 nucleotides indicated that the left terminus of EBV must be blunt. The right terminus showed a ladder of three bands for both the 3′- and 5′-labeled strands. This minor variability of 3 bases at the right end is in agreement with our cloning and sequencing results for the right terminus. Bands of corresponding relative intensities have a size difference of 7 instead of the expected 6 nucleotides. The 3′-labeled strand is longer by one base (3′ right, 117

bases). Therefore, the right end seems to have a single-base extension.

The results of sequencing the cloned terminal fragments and of labeling the native genomic termini are compared in Fig. 6. The termini of EBV DNA have different end structures: the left end is blunt, whereas the right end probably has a 3′ overhang of a single G. In the case of the right cloned terminal fragments, the treatment of virion DNA with T4 DNA polymerase has removed the single-base extension at the 3′ end by the exonuclease activity of the enzyme. Thereby, the cloned right termini appeared to be truncated by a single residue compared with the right end of the native virion DNA. The heterogeneity of 3 bases found in the right-end clones is not the result of a cloning artifact and was confirmed by the labeling experiment. It seems that the right end of EBV is generated with less specificity than the left end. The results obtained with two completely independent approaches are identical. This coincidence indicates that the complicated terminal structures of EBV virion DNA, including the duplication of the 11-bp elements and the apparent translocation of the 9-bp element, exist in virion DNA.

One TR element is sufficient and essential for DNA packaging by EBV. The determination of the terminal sequences of EBV DNA supplied us with enough information about the actual cleavage site to study the TRs functionally. Such an analysis should lead to the identification of the *cis*-acting signals essential for cleavage and packaging of EBV DNA.

A transient packaging assay that allows recombinant plasmid DNA to be tested for packaging by the endogenous EBV DNA was developed. The recombinant plasmid DNA to be tested and a BZLF-1 gene expression vector were cotransfected into
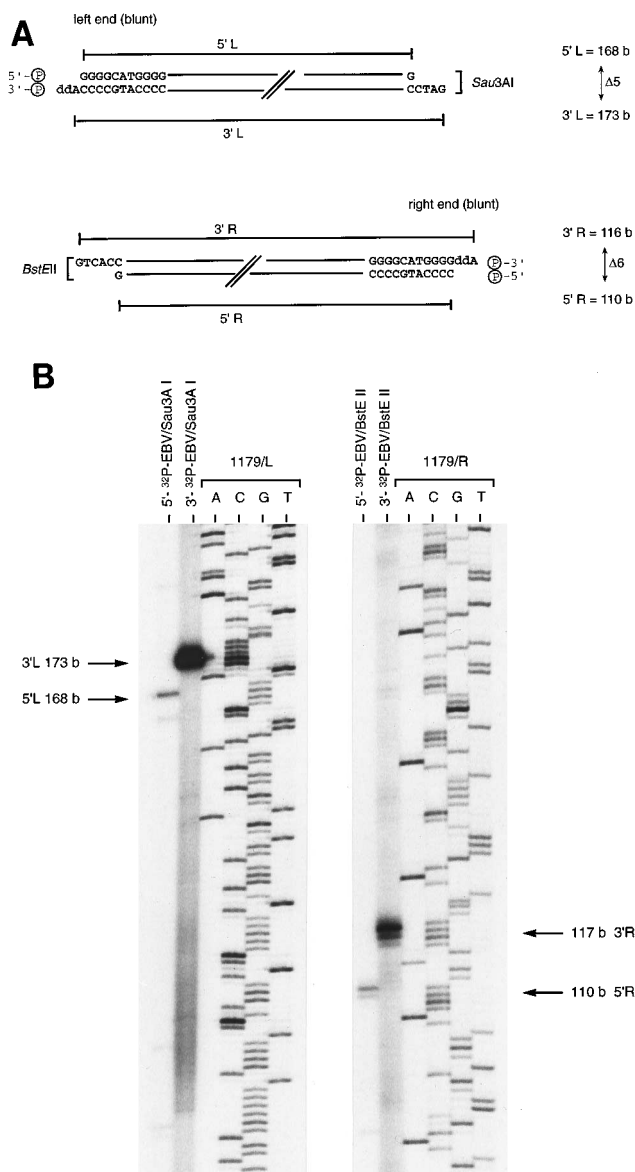
FIG. 5. Determination of the terminal structures of EBV DNA. (A) The predicted sizes of the 5′- and 3′-labeled single-stranded terminal fragments in the case of blunt termini. (B) Autoradiography of the sequencing gels. For analysis of the left (L) terminus, the 5′- and 3′-labeled Sau3AI EBV DNA fragments were loaded separately next to the sequencing ladder of the corresponding sequence of the cloned left terminus. Various exposures of the sequencing gel allowed the exact determination of the fragment lengths. The short exposure of the gel confirmed that the 3′-labeled left fragment consists of a single band. The 5′- and 3′-labeled left terminal fragments (5′L and 3′L) showed the lengths expected for blunt left termini. Similarly, the 5′- and 3′-labeled BstEII fragments of the right terminus were loaded next to the sequencing ladder of the cloned right terminus. A ladder of three bands represents the heterogeneity of 1 to 3 bp at the right terminus. The 5′-labeled top fragment (5′R) had the expected length of 110 bases, but the 3′-labeled fragment (3′R) was longer by one nucleotide (117 bases), indicating that the right terminus has a 3′ single-base overhang. The stronger intensities of the 3′-labeled fragments show that 3′ labeling had occurred with higher efficiency than 5′ labeling. Plasmid p1179 contains one TR element and was sequenced with two primers (1179/L and 1179/R) at the region of the 11-bp element to generate size markers.

a B-cell line latently infected with EBV (16). The lytic cycle of the virus was induced by expression of the BZLF-1 gene, and the viral capsid DNA was prepared and analyzed after 4 days. Plasmids p554 and p588 contain the fused termini with four TRs and oriLyt, the origin for lytic replication. Since their packaging has been shown previously, they were used as positive controls (10).

To determine whether one TR element is sufficient for packaging, we cloned a single copy of the TRs into a vector containing oriLyt. The cloning strategy is shown in Fig. 7A. To investigate the significance of the position of the 11-bp element in a single TR copy, two fragments were subcloned either with Sau3AI, so that the 11-bp element was positioned in the center of the repetitive sequence, or with EcoNI, which positions the 11-bp element more to the end of the TR sequence.

Restriction analysis of the encapsidated DNA should lead to the detectable fragments shown in Fig. 7B. After lytic replication, the concatemeric plasmid DNA is processed and packaged as multimers of the size of the EBV genome (data not shown and reference 22). Cleavage of these linear molecules of approximately 170 kbp with restriction enzymes will lead to internal fragments containing the fused TRs. In addition, terminal fragments are produced by the terminase activity and cleavage with restriction enzymes.

The positive control p554 showed the expected fusion fragment of 15 kbp and the terminal fragments (9 to 11 kbp) that are identified in Fig. 8. No fragments were seen with the negative control plasmid p562.3, since this plasmid lacks the TRs and therefore was not encapsidated. The positive control p588 also showed the fusion fragment (16 kbp) and the terminal fragments (3.3 to 4.8 kbp), of which only the top fragment with four TRs is clearly visible in Fig. 7C. Both p1187 and p1188 were packaged, although less efficiently than the plasmids with four TRs. The position of the 11-bp element within the TR sequence had only a slight effect on the efficiency of packaging.

The endogenous EBV of the cells was always packaged along with the test plasmids but was not detected with the vector probe (pUC18). As an internal control for the efficiency of DNA transfections, packaging, and equivalent preparations of the DNA samples, the Southern blots were stripped and rehybridized with EBV(B95-8) DNA. On the basis of these experiments, we consistently found that the plasmids p1187 and p1188 were less efficiently packaged than the positive controls p554 and p562.3, which is more evident in Fig. 8B (see below). Although the number of TR copies determines packaging efficiency to a certain extent, a single TR copy is sufficient and essential for processing and packaging of concatemeric plasmid DNA by EBV.

A region of 159 bp contains the cis-acting signals essential for cleavage and packaging. To identify signals important for packaging within the TR sequence, we constructed seven deletion mutants based on plasmid p1187 (Fig. 8A). These mutants were tested for packaging, and the result of the experiment is shown in Fig. 8B. Four mutants, p1438.1 to p1438.3 and p1438.7, were packaged with the same efficiency as p1187. The sequences that were deleted in these constructs do not contain signals required for encapsidation. Packaging of plasmid p1438.6 was greatly reduced, but a weak band representing the fusion fragment of 13 kbp was visible after longer exposure of the Southern blot. Plasmids p1438.4 and p1438.5 were not packaged, since their fusion fragments could not be detected at all. The signals required for processing and packaging of plasmid DNA by endogenous EBV must lie within the 159-bp sequence which had been deleted in these plasmids. The
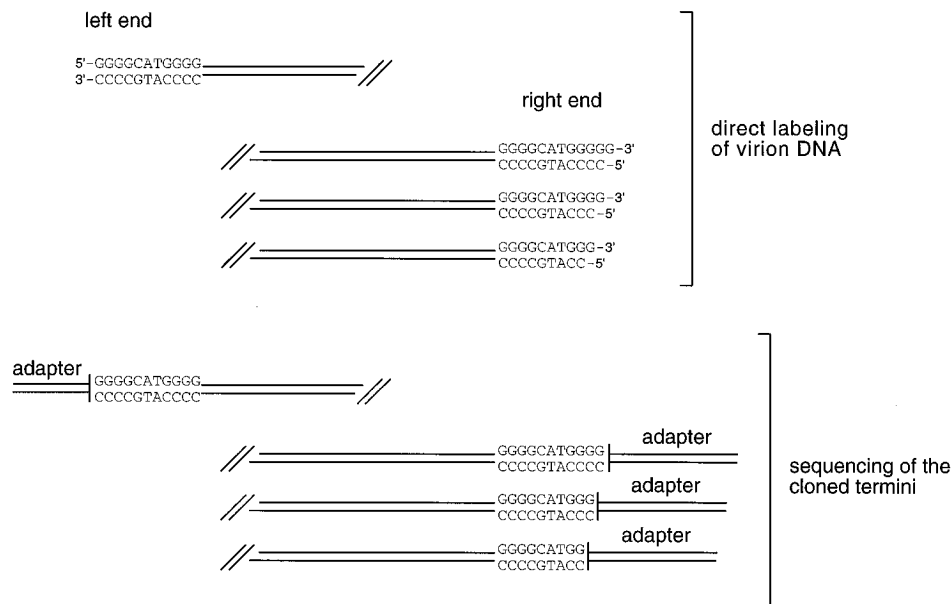
FIG. 6. The terminal structures of the linear EBV genome. The results of the sequence analysis of the cloned termini and analysis of the single-stranded terminal virion DNA fragments are compared. The left terminus is blunt and ends with the 11-bp element. The heterogeneity of 3 bases observed in the clones containing the right end could be confirmed by the labeling experiments with virion DNA. The size difference of the right single-stranded fragments indicates that the right terminus has a 3′ overhang of 1 base, which probably is a guanosine.

159-bp sequence includes the 11-bp element and the EBV equivalents of the putative packaging signals, pac1 and pac2, the only conserved regions found at the termini of herpesviruses so far (Fig. 9). Plasmids p600 and p612 contain the TRs and differ only in the orientations of *oriLyt* to the TRs. Both were packaged with equal efficiency, showing that the orientations of the TRs relative to *oriLyt* have no effect on the packaging event (Fig. 8B).

DISCUSSION

This study was performed to learn about the genomic termini of EBV virion DNA. Several structural elements which constitute the EBV termini could be identified. An 11-bp element with direct orientation was found at both termini in all sequenced clones. In addition, the right end has acquired a 9-bp element that seems to originate from the ultimate left boundary of the unique segment of EBV DNA. In a comparison of terminal sequences found in the clones with the sequence of the circular genome (1), it appears as if the ends display a 20-bp overlap. These sequence structures really exist at the native genomic termini, since they could be confirmed by measurement of the sizes of small labeled terminal fragments. Our findings indicate that the hypothetical terminase does not generate the termini during lytic replication by a simple cut as has been proposed for other herpesviruses (2, 8, 15, 23). The mechanism is more complex and may involve recombinational events.

Comparison of the TR elements of the B95-8 and the Namalwa strains of EBV revealed that the 9-bp element is an integral part of each TR element in Namalwa EBV but is present only once within the unique sequence adjacent to the TRs (Fig. 4) (13). This observation could be confirmed by sequencing of the cloned TR elements derived from B95-8 DNA which were used in this study (data not shown). According to our results, the 9-bp element was found attached to every right terminus. It appears, however, that the 9-bp ele-

ment does not have to be present in *cis* for the plasmids to be packaged. Plasmids p1187 and p1188 carry only one TR element and consequently lack the 9-bp element (Fig. 7). Either the 9-bp element is not required for cleavage and packaging, or it may have been rescued by a recombination event with the endogenous EBV in the B cell during lytic replication. Alternatively, it is possible that the 9-bp element is needed for a different process, like the circularization of the EBV genome, but not for cleavage and packaging, which alone were measured in our transient packaging assay.

The left terminus of EBV is blunt, whereas the right terminus probably carries a single unpaired G. Most herpesviruses studied so far contain complementary single-base 3′ extensions, indicating that the linear virion DNA could circularize by base pairing after infection of the host cells (2, 4, 8, 15, 23). Like pseudorabies virus with its blunt L terminus and 2-base extension at the S terminus (11), EBV has two different end structures that make circularization by base pairing and ligation improbable. In addition, the duplicated 11-bp elements at the termini of EBV must be removed upon circularization. A recombination event could expel the duplicated 11-bp element during recircularization to prevent it from becoming amplified with each virus generation. Otherwise, viral propagation would lead to an unlimited gain of redundant sequence elements at the viral termini. A highly conserved cellular protein has been found to bind to the termini of EBV in vitro, and its binding site spans the 11-bp element. It has been suggested that the protein is involved in such recombination processes, which remains to be demonstrated (21).

One TR element is sufficient for packaging, and deletion analysis identified a 159-bp sequence that includes all the requirements for processing and packaging of plasmid DNA. In Fig. 9, the terminal sequences of EBV and herpes simplex virus type 1 (HSV-1) are compared. The only two homologous sequences found at the termini of herpesviruses are the packaging signals pac1 and pac2 (5). The packaging signals are lo-
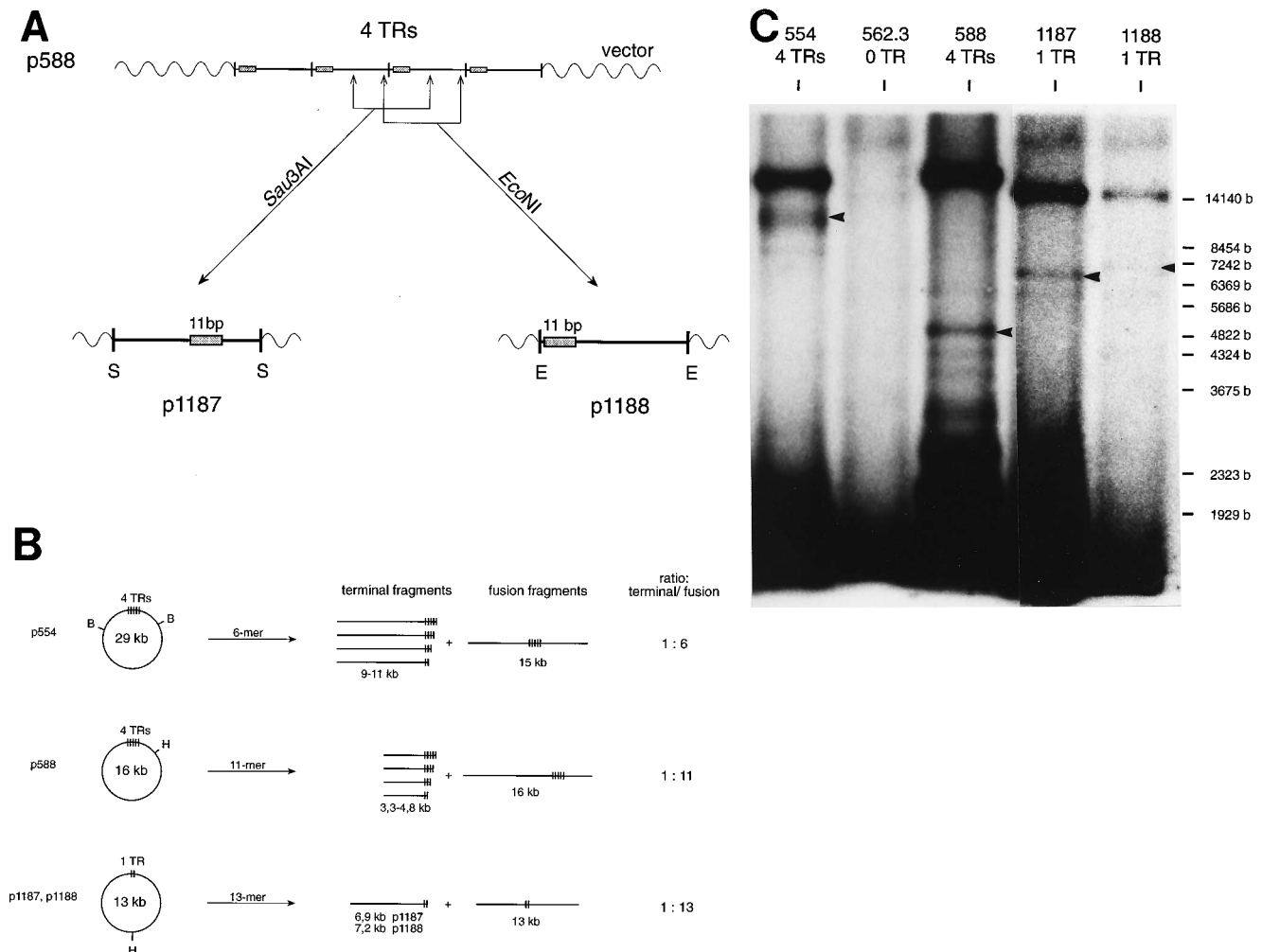
FIG. 7. One terminal repeat is sufficient and essential for packaging. (A) The strategy for cloning a single copy of the TRs is shown. Plasmid p588 contains the fused termini of EBV DNA with four TRs and the essential signals for replication, *oriLyt* and *oriP*. Two different enzymes were used for removing the repetitive sequence of 538 bp containing different positions of the 11-bp element. *Sau*3AI produces a fragment with the 11-bp element positioned in the center, and the *Eco*NI fragment contains the 11-bp element at the end. Both fragments were cloned into plasmid p562.3. The resulting plasmids, p1187 and p1188, contain one TR element with different positions of the 11-bp element. (B) Restriction analysis of encapsidated plasmid DNA. Integral numbers of plasmid molecules are packaged as linear multimers of ca. 170 kbp. In addition to the fusion fragments, the restriction enzyme cleavage of these molecules should also generate terminal fragments resulting from the action of the hypothetical terminase that cleaves the DNA within the TRs. Presumably, the plasmid p554 is packaged as a hexamer. Cleavage with *Bam*HI should lead to a ladder of terminal fragments (9 to 11 kbp) and to two fusion fragments, of which only the 15-kbp fragment with the TRs is detected with the pUC18 probe. *Hin*dIII restriction of p588, packaged as an 11-mer, generates a 16-kbp fusion fragment and terminal fragments of between 3.3 and 4.8 kbp. Plasmids p1187 and p1188 are probably packaged as 13-mers; their fusion fragments should have a size of 13 kbp, and the terminal fragments should have sizes of 6.9 kbp (p1187) and 7.2 kbp (p1188). (C) The transient packaging assay (see Materials and Methods) was used to analyze the packaging of plasmids p554, p562.3, p588, p1187, and p1188. The DNAs isolated from the virion preparations were cleaved with *Hin*dIII or *Bam*HI, separated by gel electrophoresis, transferred to nylon filters, and hybridized with pUC18 DNA. The autoradiography of the blot is shown. The positive control plasmids p554 and p588, containing four TRs, are packaged and show the predicted sizes of the fusion fragments and the terminal fragments (Fig. 7B) which can be detected with this probe. The negative control p562.3 lacks the TR elements and was not packaged. Both plasmids with a single TR element were packaged, and the fusion fragments of p1187 and p1188 could be detected. The terminal fragments of the plasmids are indicated (arrowheads), but the terminal fragment of p1188 was clearly visible only after longer exposure (data not shown).

cated at conserved distances from the actual ends of the genome (41 to 47 bp for pac1 and 20 to 35 bp for pac2) among different herpesviruses. It has been proposed that the cleavage-packaging machinery is directed to defined distances from the packaging signals. Alignment of the terminal sequences of EBV and HSV-1 revealed that the spacing between the packaging signal homologous regions and the genomic ends of EBV is similar to those of HSV-1 and other herpesviruses (Fig. 9). The packaging signals as well as the 11-bp element are included in the 74-bp sequence region that was deleted in the nonfunctional TR mutant in p1438.5. The other mutant impaired for cleavage or packaging, p1438.4, contained the pack-

aging signals and the 11-bp element. Thus, it is very likely that there are additional sequence elements adjacent to the packaging signal regions and the cleavage site itself which are required for EBV processing.

The generation of the termini of EBV DNA can be explained without the need for recombination by a model suggested for the resolution of concatemeric replication intermediates of bacteriophage T7. In this case, the concatemers are cleaved at two specific staggered points to yield 5′ overhangs, which are then filled in by repair synthesis of the polymerase. Similarly, two single-stranded cleavages, one at the bottom strand on the right side of the 11-bp element located within the
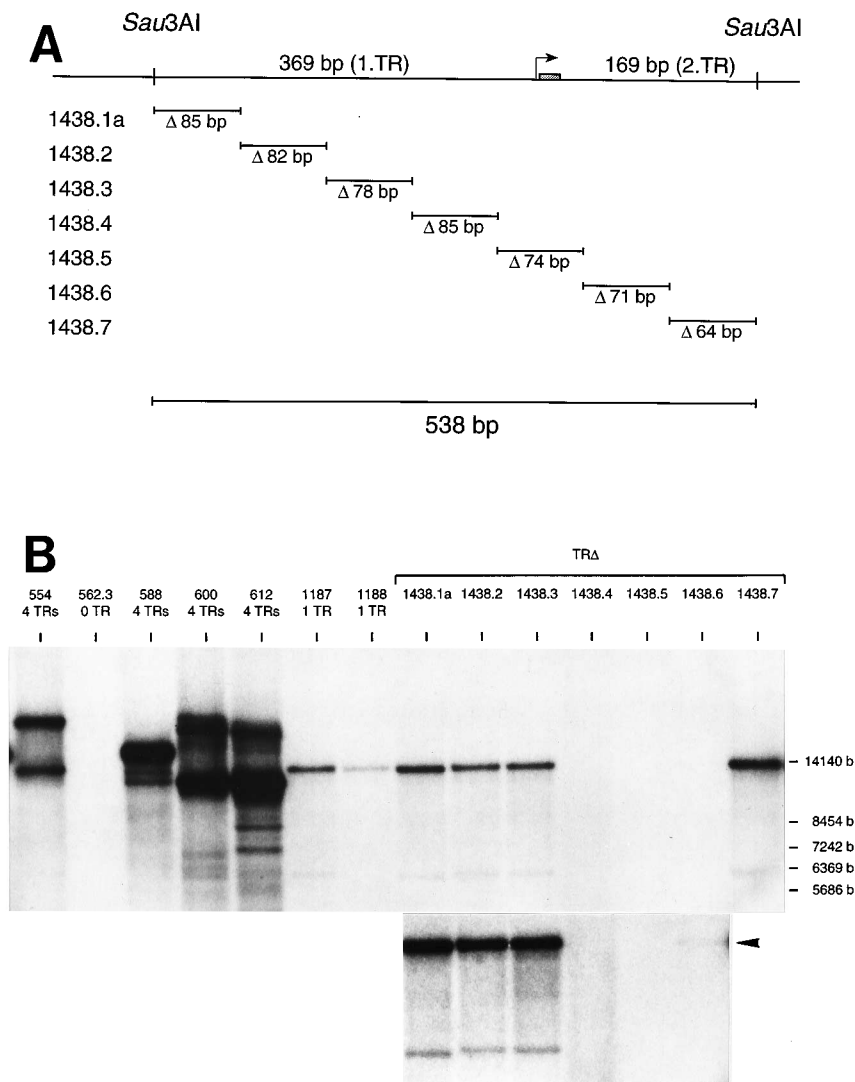
FIG. 8. Deletion analysis of the TR sequence. (A) Seven deletion mutants were constructed by in vitro oligonucleotide-directed mutagenesis of the 538-bp TR sequence of plasmid p1187. Mutants p1438.1a to p1438.7 and the sizes of the deleted sequences are depicted. A detailed description of the deletion endpoints in the individual mutants can be found in Materials and Methods. The shaded rectangle represents the position of the 11-bp element shown in Fig. 4. (B) Seven deletion mutants were tested for packaging. The plasmids analyzed in Fig. 7C were used as controls. Two plasmids, p600 and p612, containing the four TRs and *oriLyt* in different orientations, are packaged with similar efficiencies. Mutant plasmids p1438.1a, p1438.2, p1438.3, and p1438.7 were packaged. The packaging of p1438.6 was greatly reduced, but a weak band (13 kbp) could be detected after longer exposure (arrowhead) (shown below). No packaging was seen for the mutants p1438.4 and p1438.5. The 159-bp sequence deleted in these two plasmids (85 bp from p1438.4 and 74 bp from p1438.5) contains the essential signals for cleavage and packaging of plasmid DNA and corresponds to the nucleotide coordinates 170508 to 170666 of the EBV prototype strain B95-8.

unique leftmost sequence of EBV and one at the top strand on the left side of any 11-bp element, would provide staggered ends which could be filled in to yield the EBV termini (Fig. 4). This model is hampered by the observation that the copy number of TRs at the right end is variable (Fig. 1), which could not be easily explained by such a staggered-cut repair model. Therefore, we favor a recombination model to explain the formation of the EBV termini.

The termini of many herpesvirus DNAs have been shown to be involved in recombination processes. The ability to induce recombination was most intensively studied with the *a* sequence of HSV-1 (6, 14, 20, 25). A specific signal for recombination could not be found at the termini of HSV-1 (19). However, the termini contain GC-rich sequences with a large amount of small oligo(dG) and oligo(dC) repetitive elements that are related to the immunoglobulin heavy-

chain sequences involved in class switch recombination (7, 18). It has been suggested that these GC-rich sequences mediate the inherent recombinogenic activity of the *a* sequence. Although it is speculative, the class switch-like GC-rich sequences, which are an integral part of the class switch-like GC-rich sequences present within the terminal repeats of EBV and the terminal structures of other herpesviruses, may be able to interact with the recombination machinery of the host cell to perform essential viral processes. In theory, such recombination processes during lytic replication of the EBV genome could lead to the duplication of the 11-bp element and the appearance of the 9-bp element at the right end. The fact, however, that all cloned termini showed the same terminal sequences indicates that such a mechanism must be highly specific and efficient. In addition, the two different end structures of EBV indicate that the genome circularizes by a mech-

left terminus:

```
                                                     pac2                          pac2
                                                      An                         GC-motif
HSV-1 (KOS)   5'- AGCCCGGGGCCCCCCGCGGGCGCGCGCGCGCGC  AAAAAA  GGCGGGCGGCGGTCCGG   GCGGCGC ...
              3'-GTCGGGCCCCGGGGGGCGCCCGCGCGCGCGCGCG  TTTTTT  CCGCCCGCCGCCAGGCC   CGCCGCG ...
                                33 bp
                             _____27 bp_____
EBV (B95-8)        5'-GGGGCATGGGGGGCCGCGCATTCCTGG  AAAAA  GTGGAGGGGGCGTGGCCTTCCCCC  GCGGCCC ...
                   3'-CCCCGTACCCCCCGGCGCGTAAGGACC  TTTTT  CACCTCCCCCGCACCGGAAGGGGG  CGCCGGG ...
                             11 bp
```

right terminus:

```
                    _____pac1_____
                    Cn        An-motif     Cn
       ...CCCCCCCC  AAAACACA  CCCCC  GGGGGTCGCGCGCGGCCCTTTAAAGGCGGGCGGCGGGCC-3'   HSV-1 (KOS)
       ...GGGGGGGG  TTTTGTGT  GGGGGG CCCCCAGCGCGCGCCGGGAAATTTCCGCCCGCCGCCCCG -5'
                                      45 bp
                            _____42 bp_____
       .....CCCCCC  AGGAAAGA  CCCCC  GGGGGGCATCGGGGGG  GTGTTGGCG  GGGGCATGGGGG-3'  EBV (B95-8)
       .....GGGGGG  TCCTTTCT  GGGGG  CCCCCCGTAGCCCCCC  CACAACCGC  CCCCGTACCCC-5'
                                      9 bp        11 bp
```
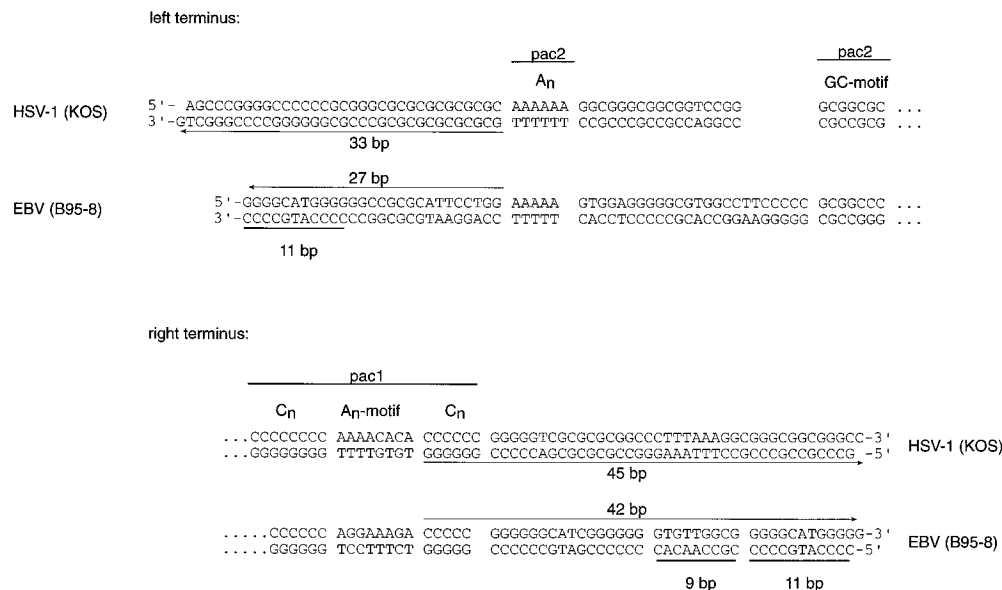
FIG. 9. Comparison of the native genomic termini of EBV and HSV-1. The terminal sequences of EBV(B95-8) and HSV-1(KOS) were aligned (24). The packaging region homologies (A_n, C_n, A_n motif and GC motif), the 11-bp elements, and the 9-bp element of EBV DNA are indicated. Arrows mark the distances between the A motif and the native ends of EBV and HSV-1 DNA.

anism that cannot easily be explained by a simple recombination event. It is therefore likely that EBV has acquired a specific and yet to be defined mechanism to maintain the integrity of its ends.

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